Genetic Diversity among Flue-cured Tobacco Cultivars on the Basis of AFLP Markers

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Abstract: AFLP analyses were used to assess the genetic similarity among selected accessions at the South China Tobacco Breeding Research Centre (Yunnan province, Southwest China). 154 AFLP polymorphic fragments out of 561 fragments were used to assess the genetic diversity among 28 tobacco accessions. The average number of polymorphic bands per AFLP primer pair was 15.4. AFLPs seemed to be an effective classification tools for germplasm conservation and breeding. Limited genetic variation was detected within this group of accessions. The relationship of cultivars was estimated by cluster analysis based on AFLP data.

Keywords: AFLP analysis; flue-cured tobacco; genetic diversity; UPGMA

China is the world's largest producer and consumer nation for tobacco. Flue-cured tobacco (*Nicotiana tabacum L.*) is one of the most important commercial types for tobacco production in the world. The study of the genetic diversity of flue-cured tobacco cultivars is important not only for germplasm studies but also for the choice of parents in tobacco breeding. Morphological, karyotypical and physiological characters have already been used to study the genetic diversity of flue-cured tobacco germplasm (Goodspeed 1945; Zhang 1994; Zhang *et al.* 2005). However, morphological characters usually vary with environments, the number of karyotypical characters is limited, and the study of genotypic diversity based on isozyme variation is restricted to a few polymorphic enzyme systems encoded by a small number of loci (Lu 1997). These methods have been improved greatly by new molecular marker analyses such as restriction fragment length polymorphism (RFLP), simple sequence length polymorphism (SSLP), randomly amplified polymorphic DNA (RAPD), randomly amplified microstallite polymorphism (RAMP) and amplified fragment length polymorphism (AFLP) (Botstein *et al.* 1980; Williams *et al.* 1991; Zhang *et al.* 2008; Liu *et al.* 2009; Mo *et al.* 2009).

AFLP (amplified fragment length polymorphism) is one of the genetic fingerprinting techniques suitable for the genetic evaluation of flue-cured tobacco. Compared with RFLP, RAPD, or microsatellites (Powell *et al.* 1996; Russell *et al.* 1997; Pejic *et al.* 1998), AFLP is more accurate and reproducible, and has been established as a reliable and efficient DNA marker system. AFLP markers have been extensively used for genetic diversity studies in different plant species (Erschadi *et al.* 2000; De Riek *et al.* 2001, Ni *et al.* 2006; Assefa *et al.* 2007; Bayazit *et al.* 2007; Mo *et al.* 2009). In this paper, AFLP analyses were used to assess the amount of polymorphism and to estimate relationships among flue-cured tobacco cultivars.

MATERIAL AND METHODS

Seeds of 28 accessions of tobacco were obtained from the germplasm collection of the South China Tobacco Breeding Research Centre in Yunnan province, southwestern China. The collection consists of 772 cultivars and breeding lines from different locations. On the basis of results from field trials conducted at Yuxi, Yunnan, from 1994 to 1996, the accessions with desirable agronomic characteristics, such as large leaf size, high leaf yield, low nicotine content, or resistance to various diseases or insects (Rei *et al*. 1997), were selected for evaluation in this study. These accessions represented genotypes likely to be used in future flue-cured tobacco breeding efforts in southern China. The following 28 tobacco cultivars were selected for AFLP analyses: 1. Changbohuang; 2. NC82; 3. 581; 4. Yunyan 3; 5. Jingtai 33; 6. Yunyan 86; 7. Yunyan 76; 8. Jingyehuang; 9. 82-3041; 10. Zhongyan 86; 11. Jiyan 5; 12. CV87; 13. 77809-12; 14. Qingsheng 2; 15. Yunyan 84; 16. Chunjingyan; 17. Jingxing6007; 18. RG11; 19. Zhongyan14; 20. Yunyan 1; 21. K394; 22. Xiaohuangjing1025; 23. 8813; 24. Yunyan 2; 25. CV85; 26. CV73; 27. Oxford 26; and 28. K149.

Seeds were planted in pots and plants were grown in a greenhouse at temperatures ranging from 28°C to 32°C. Forty seedlings were harvested from each accession, after germination lasting for about 20 days.

DNA was extracted from shoots by the CTAB method (Liu *et al.* 2009).The AFLP analysis was performed following the manual of the AFLP kit (Life Technologies). The DNA was digested with restriction enzymes *Eco*RI and *Mse*I. The selective amplifications were performed using the primer pairs listed in Table 1. Primers within the *Eco*RI set all included the sequence 5'-GAC TGC GTA CCA ATT C; primers of the *Mse*I set have the sequence 5'-GAT GAG TCC TGA GTA A. Restricted genomic DNA fragments were ligated to *Eco*RI and *Mse*I adapters. The pre-selective and selective amplifications were performed in a 2400 Perkin-Elmer Thermocycler. An equal volume of loading dye (95% v/v formamide and 0.08% w/v bromophenol blue in 20mM EDTA, pH 8.0) was added to each sample, which was then denatured at 95°C for 3 min and placed on ice for 2 min before loading. Amplification products were analyzed by electrophoresis in a 6.5% polyacrylamide gel. The electrophoresis parameters were set to 1500 V, 40.0 mA, 40.0 W, 50°C and the run time was set to 2.0 h. Separated AFLP products were visualized by silver staining as described in the Promega Silver Staining kit.

Each accession was scored 1 for presence or 0 for absence of each polymorphic band. Bands present in all accessions were not scored. Only bright, clearly distinguishable bands were used in the genetic analysis. Similarity matrices (data not shown) were constructed from the binary

Table 1. DNA fragment polymorphism in flue-cured tobacco cultivars as reflected by AFLP analysis

AFLP primer pair	Total bands	Polymorphic bands	Percent of polymorphism
E-ACC+M-CAG	52	12	23.07
E-ACC+M-CTG	76	26	34.21
E-ACC+M-CAT	66	25	37.88
E-ACC+M-CAA	39	15	38.45
E-ACC+M-CTT	49	3	6.12
E-AAC+M-CAT	64	11	17.19
E-AAC+M-CAG	38	1	2.63
E-AAG+M-CAA	47	3	6.38
E-AAG+M-CAG	81	26	32.10
E-AAG+M-CTT	53	32	60.38
Total	561	154	27.45
Average	56.1	15.4	

data with Jaccard's coefficients. Dendrograms were generated by the unweighted pair-group method, arithmetic average (UPGMA) algorithm as described by Sneath and Sokal (1973). The calculation of UPGMA and similarity matrices was performed by NTSYS-pc, Version 1.8.

RESULTS AND DISCUSSION

Fourteen AFLP selective primers were screened against all 28 accessions. Four primer pairs were not included in the final analysis because either the amplification profile was consistently too faint to score accurately (E-AAC+M-CGC) or only monomorphic amplification products were produced (E-AAC+M-CTG, E-ACT+M-CTC, E-ACT+M-CTG). The ten informative primer pairs used in the final analysis were E-ACC+M-CAG, E-ACC+M-CTG, E-ACC+M-CAT, E-ACC+M-CAA, E-ACC+M-CTT, E-AAC+M-CAT, E-AAC+M-CAG, E-AAG+M-CAA, E-AAG+M-CAG and E-AAG+M-CTT (Table 1). Five hundred and sixty-one fragments were scored in the assay performed using the ten primer pairs, with an average of 56.1 fragments per pair of primers used. One hundred and fifty-four fragments were polymorphic, with an average of 15.4 per reaction. AFLP-based Jaccard's similarity coefficients (JSCs) ranged from 0.12 to 0.94. At the 0.38 phenon level, four distinct clusters were apparent in the dendrogram produced by cluster analysis (Figure 1): I – included 17.86% of the accessions, II – included 46.43% of the accessions, III – included 17.86% of the accessions, and IV – included 17.86% of the accessions.

The dendrogram did not indicate a clear pattern of division among the flue-cured tobacco accessions based on the geographic origin, as seen in *Camellia sinensis* and *Potatoe* (Paul *et al.* 1997; Spooner *et al.* 1996). But those sharing common ancestry always clustered together. For example: the cultivars bred by Speight G-28 such as 82-3041, K149, K394, Zhongyan 14, CV73, CV85 and CV87 clustered together in dendrogram; Jiyan 5 and Qingsheng 2 were the offspring of Jingyehuang, bred by Changbohuang, they were clustered with

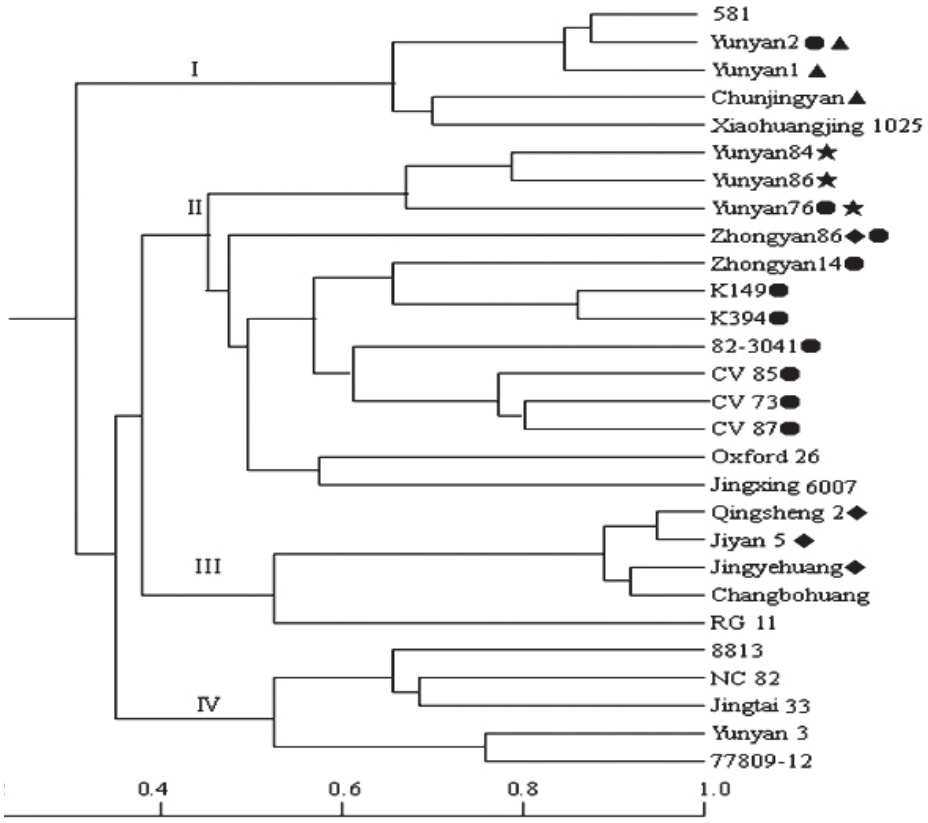

Figure 1. Dendrogram of 28 flue-cured tobacco cultivars from the cluster analysis (UPGMA) of genetic distances on the basis of AFLP data; symbols indicate ancestry of origin: ◆ = Gold dollar; ● = Speight G-28; ▲ = Jingyehuang; ★ = K326

Jingyehuang in dendrogram; Yunyan 2, Chunjingyan and Yunyan1 were derived from Gold dollar, they were clustered together in dendrogram; and one of the crossing parents of Yunyan 76, Yunyan 84 and Yunyan 86 was K326, they were also clustered together in dendrograms. Yunyan 76 is the progeny of both Speight G-28 and K326, so it was clustered with the progenies of K326 in a small cluster and it also grouped with the offspring of Speight G-28 in a bigger cluster. Yunyan 2 is the offspring of both Jingyehuang and Gold dollar, but it always clustered with the progenies of Gold dollar. May be it possesses much more genes from Gold dollar than from Jingyehuang.

The limited genetic variation was detected within this group of accessions. Of course, the genetic loci being invariant were also important for their potential to detect polymorphism in other flue-cured tobacco genotypes. In this study, the average number of bands per AFLP primer pair detected was 15.4 polymorphic and 56.1 total (invariant plus polymorphic). These results suggested the existence of limited genetic variation in flue-cured tobacco cultivars. A low polymorphism level was also revealed by physiological characters (Lu 1997) while a high polymorphism level was revealed by morphological characters (Rei *et al.* 1997). The low genetic diversity among cultivars suggested that the large differences observed in morphological traits varying with environment may result from a relatively small number of genetic differences among cultivars. In a breeding program, crosses made between genetically distant parents could give a high chance of transgressive segregation in their progeny (Wuang & Zhou 1995). Therefore, parent selection for a crossing program should be made according to their genetic distance. The results of study on the genetic diversity provide estimates on the level of genetic variation among diverse materials that can be used in assessing the purity and stability of genotypes entering into a breeding and seed multiplication program. The obtained data could also be used for reducing duplicate collections of germplasm. AFLPs appear to be valuable tools for assessing genetic distance in flue-cured tobacco.

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